TITLE: Equipment and Process for Production of pure major histocompatibility complex (MHC) antigens in human cells for broad Immunology testing and Therapeutic Use

Background

Immunology may be defined as the study of the reaction to foreign bodies including microbes, as well as macromolecules such as proteins and polysaccarides without specifying the physiological or pathological consequences of such reactions.

In the last few years recognition and defining of the multitude of genes in the human body has been proceeding at a rapid rate and major advances as the understanding of disease treatment and prevention is occurring.

The human body has a family of highly polymorphic genes called the major histocompatiblity complex (MHC) which encode trausmembrane proteins. Class I and class II MHC molecules play a central role in most if not all adaptive immune responses because class I and class II molecules present antigens to T lymphocytes. The human class I and class II molecules are known as HLA (Human Leukocyte Antigen) molecules.

Aside from their central role in directing immune responses against pathogens, the MHC class I and class II molecules are distinguished by their extensive polymorphism. This extensive polymorphism which leads most individuals to be immunologically different is what causes tissue or transplant rejection between individuals. Differences in the MHC from one individual to another

are also why some individuals are susceptible to infections while others are not. Particular MHC types are associated with autoimmune disorders such as diabetes and arthritis.

Class I and class II HLA molecules contribute significantly to autoimmunity, transplantation, susceptibility and/or resistance to infectious disease, and cancer. For these reasons a detailed understanding of the biological role of MHC proteins in immune responses is now being sought. To realize the role of HLA class I and class II molecules in human disease requires HLA class I and class II proteins. Clinical therapies to manipulate or alter immune agents which interact with HLA class I and class II molecules will also require MHC proteins.

An example of the research tests in which MHC class I and class II molecules are used include:

- MHC-peptide multimers used as immunodiagnostic reagents for disease resistance/autoimmunity
- Assessing the binding of potentially therapuetic peptides
- Elution of peptides from MHC molecules to identify vaccine candidates
- Screening transplant patients for preformed MHC specific antibodies
- Removal of antibodies from a patient

Because these studies are based around human diseases, MHC molecules produced in humans will most readily lead to accurate

research results in humans. MHC molecules produced in humans will also lead to immune based intervention and therapy in the clinic.

There are several research and clinical tests that MHC molecules can be and are used in. These include clinical crossmatch tests for solid organ and bone marrow transplantation (FACS, Elisa, columns), peptide binding tests which examine the ability of potentially therapuetic peptides to bind to various MHC class I and class II molecules (reference the work of Sette, Buus, Takiguichi, Ramnennsee), and tests for assessing the nature of immune responder cells which provide disease resistance and which drive autoimmune responses (reference Altman, McMichael).

At this point in time there is no readily available source of HLA class I or class II molecules. For research tests which require relatively pure class I or class lithe product can be made in bacterial cells. Such is the case for class I molecules used in research experiments. Although the HLA molecules produced in bacteria will not be glycosylated or have human peptides loaded into them, bacterial production is the only means by which enough class I can be produced in a pure form for experiments. Once produced in bacteria the researchers must then load peptide(s) and light chain onto the bacterial class I heavy chain. Only then can experiments be performed.

When HLA molecules are required from human cells, laboratories typically grow up large volumes (50-100 L) of static cultures (of

cells expressing multiple surface-bound HLA class I molecules) in roller bottles, following which they pellet the cells and perform established immunoprecipitation protocols to recover the multiple HLA class I molecules from cell lysates. Using these procedures, the product obtained is typically in the amount of ~300-500 μg. Note that the product obtained in this manner represents a mixture of the six different HLA class I molecules expressed on most cell lines. Interpretation of results can therefore not be certain that any particular HLA molecule is responsible for a given result. Only indirect conclusions can be reached from these mixtures.

This patent comprises equipment and processes for producing relatively large volumes of pure Class 1 molecules at a reasonable cost.

Class I human leukocyte antigens (ELLA), which are expressed by and present upon virtually every nucleated cell in the body, bind and display cytoplasmically-derived peptide antigens on the cell surface. The peptides they present are derived from either normal endogenous proteins ('self') or foreign proteins ('nonself'); foreign proteins include products of malignant transformation or intracellular pathogens such as viruses. Class I molecules thus convey information regarding the internal fitness of a cell to CD8+ cytotoxic T-lymphocytes (CTLs), which are activated by interaction with 'nonself' peptides and lyse or kill the cell presenting them. Lymphocytes are a type of white blood cell or

leukocyte that circulate in the lymph.

HLA class I molecules exhibit extensive polymorphism, which is generated by systematic recombinatorial and gene conversion events; it is for this reason that hundreds of different HLA types exist throughout the world's populations. Most people therefore differ in their MHC class I molecules.

Transplantation between individuals with different class I and class II MHC molecules leads to the production of strong immune responses. Some of these immune responses can be controlled with drugs. The immune responses that can be controlled with drugs are new immune responses, or immune responses that form after the transplant. In fact, immunosuppresive drugs are now so effective that it is becoming much more common to transplant organs that are not well matched for their MHC class I and class II molecules. However, immunosuppresive drugs cannot stop the rejection of an organ transplant when the organ recipient has circulating antibodies which recognize the organ being transplanted. A number of events can trigger the production of antibodies against MHC class I molecules in other individuals. These events include blood transfusion, pregnancy, bacterial infections, and other less understood events. So, it is not uncommon for an individual who needs a heart, kidney, lung, or liver transplant to have circulating antibodies which would immediately attack some transplanted organs. These circulating antibodies cannot be

inhibited with drugs, and transplanting an organ that is recognized by such antibodies will lead to organ failure before the transplant operation is finished.

Let's use Man X as an example. Man X needs a liver. With the powerful immunosuppresive drugs now available the transplant physician can give Man X a liver from almost any donor. The immunosuppresive drugs will mean that the liver donor does not have to be well matched because the drugs can keep the immune response from forming an attack against the nonmatched liver. However, if Man X has antibodies that recognize the liver being transplanted then he will reject the liver before it is completely sewn in.

So, in order to insure that Man X does not have antibodies against MHC class I, the hospital will take blood from Man X once a month and test it to see what MHC class I Man X does have antibodies to. If Man X has antibodies to particular MHC molecules in the organ to be transplanted then the physician will be sure not to transplant an organ with those class I molecules into Man X.

How do they do this test? They get Man X's antibodies (easy) and they see what class I molecules his antibodies recognize. The limiting reagents in all of this are Class I and Class II pure protein antigens. There is no good source of Class I and Class II to screen Man X's antibodies against at present. This patent is aimed at filling this need for Class 1 molecules.

With advances in immunosuppressor drugs transplant physicians

are now poised to do transplants between the organ to be transplanted and unmatched individuals. Less matching in now being done between organ donors and recipients than ever before. However, with less matching the odds go up that Man X will have antibodies against class I molecules; you only make antibodies against 'foreign' objects, or those that are not matched and therefore different than self. Thus, because complete organ matching is becoming less common and our test to look for preexisting antibodies against the Class I HLAs on organs to be transplanted is very important.

SUMMARY OF INVENTION

The invention encompasses production of Class I single human leukocyte antigens (HIA) of the polymorphic major histocomptability (MHC) of the immune system.

Class I molecules are ordinarily expressed on the cell in a membrane-bound form; they consist of an extracellular domain, a transmembrane domain, and a short cytoplasmic domain. We modify the molecules using PCR so that they no longer have transmembrane or cytoplasmic domains; since they no longer are anchored into the membranes of the cells expressing them, they are therefore secreted into the supernatant media surrounding the cells as they grow. We collect the molecules produced in this manner as 'harvests' from a hollow fiber bioreactor sysem.

Once we have made a 'construct' encoding a soluble molecule (this consists of taking the truncated PCR product and placing it in a DNA vector that contains a promoter which is required for expression of the molecules), the construct is introduced into a mammalian cell line so that it can be expressed; we use a human B-lymphocyte line which is mutated so that it does not express any class I molecules other than the one coded for in the construct that we incorporate into the cell. The obvious pluses of this system are that (i) the resultant molecules are produced and folded 'naturally' (since they are generated within mammalian cells, rather than made using either bacteria or insect cell lines; we

furthermore have published data confirming that the soluble molecules appear to bear functional properties identical with those of full-length, cell surface-expressed HLA molecules), (ii) due to continuous secretion large quantities of soluble molecules can be obtained with relative ease using hollow-fiber bioreactor systems, and (iii) the product yielded is significantly "cleaner" to begin with than the small quantities obtained by traditional cell lysate/immunoprecipitation protocols previously touched-upon. Edman sequencing, and mass spectrometry show that the protein content of the material we obtain from harvests consists of soluble HLA molecules of a single class I molecule.

The total steps necessary to produce cell lines prior to growth in the hollow fiber bioreactor systems is considered a 'limiting factor' in that it requires multiple factors including (i) subcloning/sequencing to obtain specific constructs, (ii) transfection/screening by ELISA of mammalian cells to obtain cell lines which have taken up the completed DNA vector for expressing the molecule, (iii) limiting dilution subcloning/screening by ELISA to obtain cell lines of maximum soluble HLA production levels, and (iv) RT-PCR/sequencing to validate cell lines before bioreactor culture

In use there is a major advantage using a single MHC molecule in a test or experiment. Experimental results are not clouded by the confounding fact that multiple MHC molecules are present. This

is a major advantage over existing tests which rely on mixtures of MHC molecules. Furthermore, the MHC molecules provided here are produced in human cells. The only existing means for producing individual MHC molecules is in non-mammalian cells. Production in human cells means that the MHC molecules produced most resemble human proteins, noting that these proteins will be used in applications for humans. For this reason MHC molecules produced in bacterial or yeast cells are not used for diagnostic or therapuetic purposes. Mixtures of MHC molecules from human cells are now the norm. We will provide individual MHC molecules produced in human cells for the following:

HLA crossmatching

- Absorbtion/removal of anti-HLA antibodies from patients
- Development of HLA presented peptide based vaccines
- Discovery of pathogen based peptide epitopes for vaccine use
- Characterization of immune effector cells

We have documented that our unique system produces individual MHC molecules in sufficient quantity for numerous experimental and clinical applications. The MHC molecules are produced from human cells and in all measurable ways they resemble surface bound MHC molecules found on healthy human cells. Chaperone interaction, peptide loading, and antibody reactivity are all normal.

DETAILED DESCRIPTION OF THE INVENTION

We will describe the invention by outing steps that we currently use to produce the pure protein antigens from single Class I HLAs.

In Step 1 we may start with an EBV (Epstein-Barr virus) transformed cell line containing the multiple alleles or forms of the MHC of a human DNA strand. We start at this point because this is readily commercially available. We could start from normal mammalian DNA and produce the virus transfomed cell line or an immortalised cell line by known methods.

Using PCR we grow the cell line and in Step 2 we spin down cells and extract the total RNA using Qiagen RNA extraction kit or similar separation means.

In Step 3 cDNA is formed from Step 2 product using an Amersham-Pharmacic Biotech Kit or similar equipment and reverse transcriptase to form complementary DNA or cDNA.

In step 4 product from Step 3 is treated with a pfu polymerase enzyme or similar enzyme and primers to cut off a sequence which encodes cytoplastic and transmembrane domains there by truncating said molecules so that the normally surface expressed peptides will be secreted in the surrounding solution. If we wish the secreted peptides to have histidine tails another primer such as 3PEI-His is used. Tails other than the histadine tails shoud be equally useful. The truncated product may be purified using a Qiagen

PCRPurifIcation kit or similar apparatus

In Step 5 restriction enzymes such as EcoRl and Xbal cut the product to form single strands and form the pcDNA3.1 vector.

In step 6 using a T4 enzyme ligase insert the PCR product into the vector.

In step 7 transform JM 109, a strain of E-coli, using the ligated product and vector from step 6; thus causing the ligated vector to enter the JM109 bacteria. Other bacteria should work essentially as well.

In step 8 plate out the transformed JM109 on 96 cell LB/ampicillin plate or similar equipment and grow for about 12 hours. Only antibiotic resistant colonies should grow.

In step 9 pick out antibiotic resistant colonies and grow in LB/ampicillin media while shaking at 300 rpm for approximately 18 hours and make a small volume of glycerol stocks of each and allow to stand overnight.

In step 10 using a portion of the glycerol stock, extract the vector or plasmid containing the PCR product insert using a Promega Wizard mini prep kit. This extraction occurs by the vector sticking to the kit membrane where it is then water washed.

In step 11 using the extracted vector and using restriction enzymes such as CcoR and Xbal carry out said restriction digest on an ethidium bromide agarose electrophororesis gel. The ethdium bromide is an intercalating agent and allows us to visualize the

DNA in the gel under ultraviolet light. Grow to bring up the concentration of the vector to 9.5μg/10μl.

In step 12 using step II product and primers such as BGH, 3S, and T7 to cut double DNA strands and using an Amersham Pharinacia Sequencing kit and sequencing gel analyze the data to make certain the insert has no errors. If errors are found go back to the glycerol solutions and repeat the steps.

In step 13 pick a good clone from step 12 and streak an LB/ampicillin plate using the using the stored glycerol stock of bacteria and grow overnight; pick a colony from the plate, place in 45 mls of LB/ampicillin media and grow for 16 hours while shaking at 300 rpm. Actual timeand shake rate may be varied.

In step 14 using a Qiagen Midi kit or similar kit extract the plasmid containing the PCR product insert from the 45 mls of solution.

In step 15 using cell line 721.221 or an immortalised cell line that lacks expression of Class 15 MHCs grow using PCR until it is in log phase.

In step 16 electroporate the plasmid or vector containing the PCR product insert into the 721.221 cells and using G418 or similar antibiotic choose electroporated cells that are resistant to the antibiotic proving the cells contain the vector with the insert therein.

In step 17 using electroporated cells serially dilute in an

agarose plate to a point where there is one cell per cup, grow and using ELISA Assay determine which cells are producing the most soluble HLA.

In step 18 grow most prolific cells in complete media in roller bottles or similar containers. Where only small amounts are needed the small amounts may be harvested from the roller bottles or similar equipment. Inoculate a hollow fiber reactor unit with the product.

In step 19, product from the roller bottles is fed counter current to harvest media in the hollow fiber bioreactor unit for continous large scale production of desired secreted antigen Oxygen, glucose and carbon dioxide are fed into the temperature controlled circulating stream with feed rate, circulating rate, oxygen, glucose, a nd carbon dioxide and pH all controlled to to harvest a concentration of about 3μg/ml of class I pure protein antigen.